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Semi-preparative purification of an endogenous ligand for brain serotonin-2 receptors by coil planet centrifuge counter-current chromatography

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ABSTRACT

A horizontal flow-through coil planet centrifuge equipped with a rotatory frame holding three sets of composite column assemblies was used for purification of an endogenous ligand (ketanserin binding inhibitor) for the [^{3}H]-ketanserin (^{3}H-KET) recognition site. The protein mixture containing the endogenous material was successfully resolved by using a two-phase solvent system consisting of 95% ethanol–31.5% ammonium sulphate (1:2). The active fractions on ^{3}H-KET binding obtained after counter-current chromatography (CCC) were further purified through a C_{18} μBondapak reversed-phase high-pressure liquid chromatographic column. The introduction of this advanced CCC technique represents an important step in the application of CCC for the separation of polar proteins from protein mixtures.

INTRODUCTION

In recent years, counter-current chromatography (CCC) has been used mostly for purification of substances with relatively high hydrophobicity [1]. Only recently, however, this technique has been applied to highly polar biological mixtures such as endogenous substances in brain [2] and anti-trypanosomal factor from *Pseudomonas fluorescens* [3]. Purification of proteins and peptides from brain and other tissues has been carried out classically using a wide variety of chromatographic methods including gel filtration, ion-exchange chromatography, affinity chromatography, CCC, electrophoresis, high-performance liquid chromatography (HPLC), etc. [4]. The recent introduction of an advanced centrifugal CCC technique in our laboratory represents an important step in the application of CCC to the separation of protein mixtures.

Previous studies from our group, as well as from other laboratories [5–7], have led to the conclusion that serotonin might not be the primary transmitter for the so-called serotonin-2 (5-HT-2) receptor. If this assumption holds true, an endogenous physiological chemical signal to be transduced by the 5-HT-2 receptor might exist.

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This endogenous ligand would function as a modulator or co-transmitter of the serotonergic receptors located at the post-synaptic site.

Binding techniques provide an easy and sensitive approach to study ligands for the 5-HT-2 recognition site and may be used to monitor the purification of endogenous putative ligands for 5-HT-2 receptors. Using [^{3}H]ketanserin (^{3}H-KET) as a radioligand probe to label the 5-HT-2 site, it was possible to extract, isolate, and purify ketanserin binding inhibitor (KBI), an endogenous compound that selectively inhibits ^{3}H-KET binding.

EXPERIMENTAL

Materials

Frozen rat brains were obtained from Zivic-Miller (Allison Park, PA, U.S.A.). Ammonium sulphate was purchased from Baker (Phillipsburg, NJ, U.S.A.). Glacial acetic acid and organic solvents were obtained from Aldrich (Milwaukee, WI, U.S.A.). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.). Bio-Gel P-10 resin was purchased from Bio-Rad (Richmond, CA, U.S.A.). The C_{18} μBondapak HPLC column was purchased from Waters (Milford, MA, U.S.A.). Ketanserin was obtained from RBI Labs. (Natick, MA, U.S.A.). ^{3}H-KET was purchased from New England Nuclear (Wilmington, DE, U.S.A.).

Protein purification

Routinely 360 g of rat brain were homogenized in 6 volumes of 0.1 *M* hot (80°C) acetic acid and centrifuged at 27 000 *g* for 15 min at 4°C. The clear supernatant was lyophilized and resuspended in 15 ml of 20% methanol–0.1 *M* acetic acid solution and applied to a Bio-Gel P-10 column (60 cm × 2.5 cm) with an eluting solvent consisting of 20% methanol–0.1 *M* acetic acid. Fractions of 2 ml were collected and aliquots of 50 μl were lyophilized and resuspended inTris–HCl buffer at pH 7.4 to determine its inhibitory activity of ^{3}H-KET binding. The fractions that inhibited ^{3}H-KET binding were then pooled and lyophilized. The material was resuspended in 0.1 *M* acetic acid and precipitated with 60% ammonium sulphate. After a 30-min stirring period, the sample was centrifuged at 40 000 *g* for 30 min and the supernatant was desalted by eluting a C_{18} Sep-Pak column with a 0.1 *M* TFA–60% acetonitrile solution and lyophilized. This material was injected directly into the CCC column.

Radioligand binding

^{3}H-KET binding was assayed on twice washed cortical synaptic membranes using 0.5 n*M* radioligand in 50 m*M* Tris–HCl buffer, pH 7.4. Incubation was performed at 4°C during 75 min. The reaction was stopped by filtration as previously described [6,8]. Non-specific binding was determined using 1 μ*M* ketanserin.

Counter-current chromatography

Purification of KBI was carried out using a horizontal flow-through coil planet centrifuge equipped with three column holders (Model CCC-1000) (Pharma-Tech Research Corp., Baltimore, MD, U.S.A.). The rotatory frame of the apparatus holds three sets of composite column assemblies. Each assembly consists of a series of coiled columns which were prepared by winding two layers of PTFE tubing (1.6 mm I.D. ×

0.5 mm wall) onto ten stainless-steel rods (10 cm × 6.35 mm O.D.). The three composite columns were connected in series and mounted symmetrically on the column holder at a distance of 7.6 cm from the holder axis. The total capacity of the coiled column measured 150 ml. The two-phase solvent system was prepared by mixing 95% ethanol and 31.5% ammonium sulphate solution at a 1:2 volume ratio. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use. In a typical experiment, the stationary phase (upper non-aqueous phase) was introduced into the column at a flow-rate of 5 ml/min using a Model 300 LC perfusion pump (Scientific Systems, State College, PA, U.S.A.). After introduction of the stationary phase, the column was then rotated at 1000 rpm, and elution with the mobile phase (lower aqueous phase) started at a flow-rate of 1 ml/min. After perfusing 30 ml of mobile phase, the sample (2 ml total volume equilibrated in the two-phase solvent system) was introduced into the column through a four-way injection valve. Fractions of 2 ml were collected in a Model FRAC-100 fraction collector (Pharmacia, Piscataway, NJ, U.S.A.). After injection of the sample, the elution continued for 180 min. The volume of the stationary phase retained in the column averaged about 26% of the total column capacity. The elution profile was monitored using a Model SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan) coupled to a Model B5117-5 I Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). A 300-μl aliquot of each fraction was desalted through C_{18} Sep-Pak columns, lyophilized, and tested for its activity on ^{3}H-KET binding.

HPLC analysis

Further purification of the material was carried out using a Spectra-Physics liquid chromatographic system equipped with a Spectra-Physics Model 8700 solvent delivery system. The proteins were detected by monitoring absorbance at 210 nm. The material from the C_{18} μBondapak column previously equilibrated with a solution of 10% methanol–0.15 *M* acetic acid was eluted with a 0–60% gradient of a solution of 70% acetonitrile–15% methanol–0.1 *M* acetic acid. Fractions of 1 ml were collected and a 200-μl aliquot of each fraction was lyophilized and tested for activity on ^{3}H-KET binding.

RESULTS

Determination of the partition coefficient (K) of KBI in different two-phase solvent systems

As shown in Table I, the *K* values of KBI were determined using four different two-phase solvent systems. For these experiments, partially purified KBI obtained after Bio-Gel P-10 and 60% ammonium sulphate precipitation was used. After desalting and lyophilization, the material was dissolved in the solvent system under study. After equilibration, similar aliquots of the upper and lower phases were lyophilized and tested for their activity on ^{3}H-KET binding. The *K* value was determined by calculating the ratio between the percentage of ^{3}H-KET specific binding displacement by the upper phase (*a*) and that by the lower phase (*b*): $K = a/b$.

The first two solvent systems tested (*n*-butanol–acetic acid–water, 4:1:5, and *n*-butanol–phosphate buffer, 1:1) showed *K* values of 0, indicating that KBI was

TABLE I

PARTITION COEFFICIENT (K) VALUES OF KBI ON DIFFERENT TWO-PHASE SOLVENT SYSTEMS

Abbreviations: ($\%I_u/\%I_l$) = percentage inhibitory activity of KBI in the upper phase/percentage inhibitory activity in the lower phase; R = retention volume; V_c = total column capacity; R_{sf} = retention volume of the solvent front.

Solvent system	Direct analysis ($\%I_u/\%I_l$)	HPLC ($\%I_u/\%I_l$)	CPC-CCC $\frac{R - R_{sf}}{V_c - R_{sf}}$
n-Butanol–acetic acid–water (4:1:5)	0	–	–
n-Butanol–2 M phosphate buffer (1:1)	0	–	–
12.5% PEG 1000 in 1.5 M potassium phosphate buffer (pH 6.9)	1.7	–	–
1-Propanol–acetic acid–43% ammonium sulphate[a] (1:1:3.5)	0.97	–	Peak A: 0 Peak B: 0.15
95% Ethanol–31.5% ammonium sulphate[a] (1:2)	0.84	0.77	Peak 2: 0.34 Peak 3: 0.46

[a] Upper (organic) phase: stationary phase.

present only in the lower aqueous phase (Table I). Evaluation of a third solvent system containing 12.5% polyethyleneglycol (PEG) 1000 in 1.5 M phosphate buffer yielded a K value of 1.7 (Table I). This result prompted us to determine the feasibility of this solvent system for CCC separation and purification of KBI. Using PEG-rich upper phase as the stationary phase and the lower phosphate buffer as the mobile phase, CCC separation yielded multiple protein peaks by monitoring the absorbance profile at 210 nm. The use of this solvent system for purification of KBI, however, was hampered by the difficulty in eliminating the PEG 1000 present in each sample which interfered with the ^{3}H-KET specific binding assay.

The fourth solvent system analyzed (1-propanol–95% ethanol–43% ammonium sulphate, 1:1:3.5) yielded a K value of 0.97. When this system was applied to CCC using the organic upper phase as the stationary phase and the lower aqueous phase as the mobile phase, the absorbance profile monitored at 210 nm showed four partially resolved peaks eluted immediately after the solvent front. The first two peaks (peaks A and B, Table I) were active on binding while the third and fourth peaks presented little activity. Graphic analysis of the partition coefficients of peaks A and B showed K values different from those anticipated by the two-phase distribution of KBI in the same solvent system (Table I). The inability of this solvent system to resolve the active peaks from the bulk of proteins eluted near the solvent front led us to discard this system.

After evaluation of the eluotropic, solubility, and hydrophilicity parameters of a series of organic solvents it was concluded that a combination of 95% ethanol and high salt concentration represented a suitable solvent system for the separation of these highly hydrophilic compounds. The solvent system formed by 95% ethanol–31.5% ammonium sulphate (1:2) yielded a K value close to 0.8. This value was

constant either after determination of the partition coefficient through direct analysis of the two-phase distribution of KBI or after injection of equal volumes of each layer of the two-phase solvent system on a C_{18} μBondapak reversed-phase HPLC column. When this solvent system was used for CCC analysis, the K values differed from those anticipated using the methods mentioned above (Table I).

The efficient separation provided by this solvent system and the reproducibility of the absorbance profiles monitored at 210 nm led us to pursue the purification of KBI using a mixture of 95% ethanol–31.5% ammonium sulphate (1:2).

Physical properties of the 95% ethanol–31.5% ammonium sulphate (1:2) solvent system

As shown in Table II, all the physical parameters so far tested indicate that the two-phase solvent system used in the purification of KBI can be included within the hydrophilic systems. Both viscosity and density values are highest among the hydrophilic solvent systems (Table II and ref. 9). Similarly, both interfacial tension difference ($\Delta\gamma$) and settling time values (T and T') are also among the highest when compared to other hydrophilic systems (Table II and ref. 9). These particular physical properties led us to choose the present CCC centrifuge instead of a high-speed CCC centrifuge because the latter would not retain this highly hydrophilic solvent system possessing a long settling time.

Purification of KBI through a coil planet centrifuge

The active material on ^{3}H-KET binding obtained after acid extraction, Bio-Gel P-10 chromatography and ammonium sulphate precipitation represents a complex mixture of proteins of molecular weight below 20 000. For separation by CCC, an amount of material equivalent to 40–60 mg of protein is injected. Consequently, a high concentration of the solute in the two-phase system considerably alters the two-phase equilibrium composition and the partition coefficient values of the sample components. This equilibrium point may change according to the amount of material injected and, in turn, this could result in changes in the elution profile. To overcome this problem, the material was dissolved with a saturating concentration of ammonium sulphate (95% ethanol–40% ammonium sulphate 1:2). In this condition, the excess of the salt was precipitated from the sample solution and eliminated by centrifugation at 9400 g for 5 min at room temperature. The resultant supernatant (2-ml volume) was injected through an injector 30 min after starting the elution of the mobile phase (31.5% ammonium sulphate). This delay in the sample injection proved crucial for the successful separation of the active material.

As observed in Fig. 1, the elution profile shows multiple UV-absorbing peaks under the conditions selected. Determination of the activity of each single fraction after desalting 300-μl aliquots on C_{18} Sep-Pak columns indicated that the fractions active on ^{3}H-KET binding do not always correspond to the UV-absorbing peaks observed in the elution profile. The pattern of inhibitory activity of the eluted material on ^{3}H-KET binding after CCC clearly shows five different peaks, indicated as peaks 1–5 (Fig. 1.).

HPLC purification of KBI after coil planet centrifuge CCC

Fractions corresponding to peaks 1–5 underwent lyophilization, and the result-

TABLE II

COMPARISON OF THE PHYSICAL PROPERTIES OF SOME HYDROPHILIC TWO-PHASE SOLVENT SYSTEMS

Abbreviations: η_u/η_l = upper phase viscosity/lower phase viscosity; η = mean viscosity of the two phases; ρ_u/ρ_l = upper phase density/lower phase density; Δ_ρ = density difference between the two phases; T = settling time after gentle mixing; T' = settling time after vigorous shaking.

Solvent system[a]	Interfacial tension, $\Delta\gamma$ (dyne/cm)	Viscocity (cp)		Density (g/cm^3)		Settling time at 22°C (s)	
		η_u/η_l	η	ρ_u/ρ_l	Δ_ρ	T	T'
n-Butanol–acetic acid–water (4:1:5)	<1	1.63/1.40	1.52	0.90/0.95	0.05	38.5	37.5
n-Butanol–acetic acid–1 *M* NaCl (4:1:5)	1	1.69/1.26	1.48	0.88/1.05	0.16	26.5	24.5
sec.-Butanol–water	<1	2.70/1.67	2.19	0.87/0.97	0.10	57.0	58.0
sec.-Butanol–1 *M* NaCl (1:1)	3	1.91/1.29	1.60	0.84/1.03	0.19	34.0	33.5
95% Ethanol–31.5% ammonium sulphate (1:2)	3	2.90/2.82	2.86	.95/1.15	.20	41.0	40.0

[a] Obtained from ref. 9.

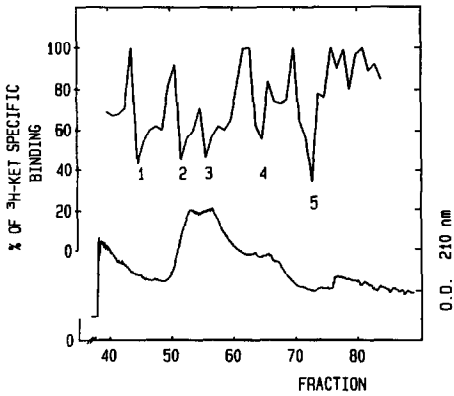

Fig. 1. Counter-current chromatography elution profile of KBI: UV absorbance and percentage inhibition of ^{3}H-KET specific binding.

ing material was redissolved in 2 ml of a 0.15 *M* acetic acid–10% ethanol mixture. Aliquots of 2–3 mg were applied to a semipreparative C_{18} μBondapak reversed-phase HPLC column previously equilibrated with a mobile phase A (0.15 *M* acetic acid–10% methanol) and eluted with a mobile phase B (0.10 *M* acetic acid–15% methanol–70% acetonitrile) gradient of 0–60% during 60 min. Evaluation of the inhibitory activity of peaks 1–5 after HPLC showed that only the material obtained from peak 2 and, to a much lesser degree, peak 3 was active on ^{3}H-KET binding. The inhibitory activity on ^{3}H-KET binding of peaks 1, 4 and 5 was no longer observed after HPLC purification. Fig. 2 shows the comparison on the elution profile and activity pattern after HPLC purification of KBI before (A) and after (B) (peak 2) CCC. A clear reduction in the absorbance peak is observed in the sample after CCC with almost no changes in the activity of the material on ^{3}H-KET binding.

DISCUSSION

The combination of different purification procedures including acid extraction, gel filtration of the supernatant and ammonium sulphate precipitation enabled us to obtain a partial purification of KBI. These high-capacity purification steps, however, did not allow enough purification on a semi-analytical or analytical low-capacity system (HPLC). At this point, the use of an intermediate capacity purification approach became necessary.

Analysis of the distribution pattern of KBI in the different solvent systems and the elution profile in CCC using the solvent system 1-propanol–95% ethanol–43% ammonium sulphate (1:1:3.5) suggested that this material had high polarity (see Results). While polymer phase systems are described as the most suitable approach for separation of hydrophilic compounds [10], difficulty in eliminating the PEG 1000 from single fractions hampered the use of this system.

The discrepancy between the partition coefficient values obtained after distribution of KBI in the two-phase solvent systems and after CCC separation reinforces the idea that *K* values in the sample mixture should be determined by HPLC analysis on each layer of the two-phase solvent system [11]. In the studies using the 95% etha-

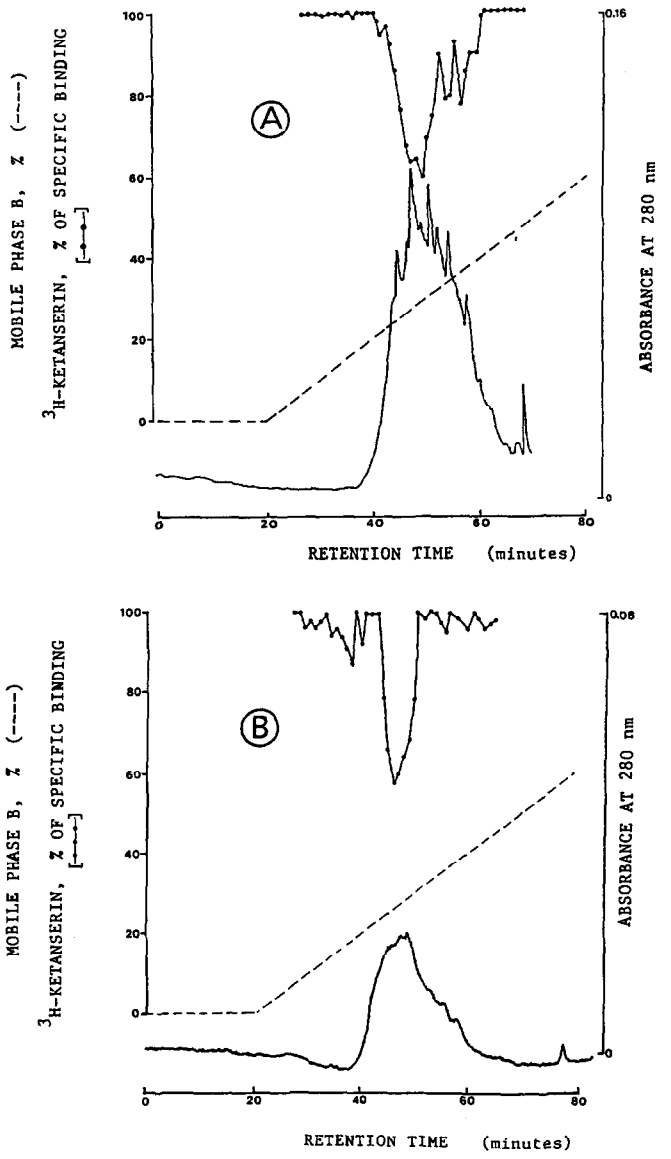

Fig. 2. Reversed-phase HPLC of KBI before (A) and after (B) counter-current chromatography: UV absorbance and percentage inhibition of ^{3}H-KET specific binding.

nol–31.5% ammonium sulphate (1:2) solvent system, however, it was observed that the K values were similar either after direct analysis of each layer of the two-phase solvent system on ^{3}H-KET binding or after analysis of the activity profile on ^{3}H-KET binding following the injection of each layer of the two-phase solvent system on a C_{18} μBondapak reversed-phase HPLC column. The inability of the reversed-phase HPLC system, under the present experimental conditions, to resolve the different

peaks of activity on ^{3}H-KET binding, as observed after CCC, could provide a rational basis for explaining the failure of HPLC analysis to provide accurate data on partition coefficient values in the complex sample mixture.

The physical properties of the 95% ethanol–31.5% ammonium sulphate (1:2) system place this two-phase solvent system in the framework of hydrophilic solvent systems [9]. However, the low interfacial tension and high viscosity make this solvent system unsuitable for high-speed CCC [12] due to the carry-over of the stationary phase from the column, as observed with the polymer phase system. The high polarity of the mixture, however, enables the separation of highly hydrophilic protein mixtures without the problems observed with the polymer phase system.

A highly effective separation was obtained when the upper (ethanol) phase was used as a stationary phase. When the lower (ammonium sulphate) phase was used as the stationary phase, fluctuations in the percentage of stationary phase retention produced inconsistent separation profiles from run to run. Further analysis of peaks 1–5 through reversed-phase HPLC revealed that only the second and, in a very small percentage, the third peak contained KBI activity. No significant KBI activity was detected after HPLC of peaks 1, 4 or 5. The ability of CCC to separate active material on ^{3}H-KET other than KBI (serotonin, etc.) provides further evidence on the efficiency of this system for semi-preparative purification of KBI. Moreover, comparison of the HPLC elution profiles before and after CCC (Fig. 2) provides reasonable information on the increase of specific activity of KBI.

In conclusion, the present semi-preparative CCC represents an important complementary approach in the purification of KBI. In this particular case, the coupling of CCC to HPLC significantly improved the purification of our material.

REFERENCES

1 N. B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography*, Marcel Dekker, New York, 1988.
2 I. Hanbauer, A. G. Wright and Y. Ito, *J. Liq. Chromatogr.*, in press.
3 T. I. Mercado, Y. Ito, M. P. Strickler and V. J. Ferrans, *J. Liq. Chromatogr.*, 11 (1988) 203.
4 M. P. Deutscher (Editor), *Methods Enzymol.*, 182 (1990) 309.
5 M. L. Barbaccia, O. Gandolfi, D.-M. Chuang and E. Costa, *Neuropharmacology*, 22 (1983) 123.
6 O. Gandolfi, M. L. Barbaccia and E. Costa, *Life Sci.*, 36 (1985) 713.
7 M. Quik and E. Azmitia, *Eur. J. Pharmacol.*, 90 (1983) 377.
8 J. E. Leysen, C. J. E. Niemegeers, J. M. van Nueten and P. M. Laduron, *Mol. Pharmacol.*, 21 (1982) 301.
9 Y. Ito and W. D. Conway, *J. Chromatogr.*, 301 (1984) 405.
10 P. Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Wiley-Interscience, New York, 1986.
11 W. D. Conway and Y. Ito, *J. Liq. Chromatogr.*, 7 (1984) 291.
12 Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.